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## **Protein aggregates modulate the texture of emulsified and acidified acid milk gels**

Perrine Gelebart, Alain Riaublanc, Marie-Hélène Famelart, Camille Jonchere, Valerie Beaumal, Marc Anton, Catherine Garnier

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31 **Keywords**

32 Protein aggregates, texture, acid milk gels, rheological measurements, whey separation, protein  
33 network, interfacial composition

34 **Highlights**

- 35 • WP addition is the most efficient way to improve texture in acid milk gel.  
36 • Fractal aggregates led to firmer and more solid-like gels with less wheying-off.  
37 • Mixed and microgel aggregates formed protein enriched gels with stable firmness.  
38 • Increased interfacial surface led to fractal aggregates-enriched interfaces.  
39

40 **Chemical compounds used in this article:**

41 Sodium Dodecyl Sulfate (PubChem CID: 3423265); Sodium Azide (PubChem CID: 33557); Nile  
42 red (PubChem CID: 65182); Alexa fluor 488 (PubChem CID: 102384756).

43 **1- Introduction**

44 The quality of fermented dairy products such as yogurts depends on a fairly complex assembly of  
45 criteria to be appreciated by consumers such as their texture, taste and health benefits (Vasbinder,  
46 Alting, Visschers, & De Kruif, 2003). How texture is perceived has a strong impact on product  
47 acceptance and sensory qualities. Texture depends on the composition of the product (protein  
48 concentration, fat content...), but also on how it is produced. Milk homogenization at different  
49 pressures is conducted to prevent fat separation, to decrease the size of fat droplets and reduce  
50 whey separation (Lucey & Singh, 1997). A large portion of whey proteins are denatured by the  
51 heat treatment also used to eliminate the potential presence of pathogenic microorganisms  
52 (Guyomarc'h, Jemin, Tilly, Madec, & Famelart, 2009; Lucey, Munro, & Singh, 1998; Lucey,  
53 Teo, Munro, & Singh, 1998).

54 Heating milk at a temperature exceeding 80 °C increases gel firmness. However, the effect of this  
55 parameter on the milk and on the acid gels strongly depends on both temperature and duration:  
56 heat treatments at very high temperatures, such as sterilization (110 °C for 30 min or 130 °C for

57 45 s) or UHT treatment (145 °C for 1 to 2 s) can change the flavor and color of milk due to a  
58 Maillard reaction (Sfakianakis & Tzia, 2014). Heat treatments applied to milk induce the  
59 denaturation of whey proteins (WP) at temperatures between 60 and 80 °C (Anema & Li, 2015;  
60 Cayot & Lorient, 1998; Chen & Dickinson, 1998; Paulsson & Dejmek, 1990). During the  
61 denaturation step, the low energy bonds that stabilize the secondary and tertiary structures of  
62 proteins are broken (hydrogen bonds, hydrophobic or Van der Waals interactions). The  
63 hydrophobic residues, present in the core of the native globular structure get exposed at the  
64 surface (Cayot & Lorient, 1998; Kella & Kinsella, 1988). As a result, at a concentration of  
65 proteins which is sufficient, hydrophobic interactions and disulfide bonds between the denatured  
66 proteins lead to the formation of aggregates. Denatured whey proteins interact with each other, or  
67 with the  $\kappa$ -casein at the surface of the casein micelle, via disulfide bridges and low energy bonds.  
68 These modifies the initial properties of proteins, such as their hydrophobicity and charge (Donato  
69 & Guyomarc'h, 2009; Mahmoudi, Axelos, & Riaublanc, 2011; Mahmoudi, Gaillard, Boué,  
70 Axelos, & Riaublanc, 2010; Vasbinder, Van Mil, Bot, & De Kruif, 2001). The interactions of  
71 whey proteins with caseins increase the hydrophobicity of the micellar surface, which has been  
72 reported to promote gelation (Lucey, Munro, et al., 1998). It also leads to an increase in gel  
73 firmness (Guyomarc'h et al., 2009; Lucey & Singh, 1997; Vasbinder et al., 2001). Furthermore,  
74 newly formed soluble aggregates can be trapped in the protein network or remain present in the  
75 aqueous phase. Heat treatment was shown to also increase whey retention capability  
76 (Guyomarc'h et al., 2009; Lucey, Munro, et al., 1998; Lucey & Singh, 1997; Lucey, Teo, et al.,  
77 1998; Vasbinder et al., 2001). Altogether, these contribute to the modification of the rheological  
78 properties of the final products and it has been a major concern for the optimization of the texture  
79 of yoghurt. The effects of heat treatment and of the addition of WP on the rheological properties  
80 of acid milk gels have already been studied (Lucey, Munro, & Singh, 1999; Lucey, Teo, Munro,  
81 & Singh, 1997) and show a strong increase in gel firmness. When adding different types of whey  
82 protein aggregates of different sizes, it was shown that the microstructural properties of acid milk  
83 gels without fat can be improved with small aggregates (Liu et al., 2016). Moreover, gels  
84 containing WP or WP aggregates reach higher G' values and gel earlier. This may be due to a  
85 greater reactivity of the -SH groups of whey proteins after denaturation, and to their interactions  
86 with caseins (Aguilera, Xiong, & Kinsella, 1993; Morand, Guyomarc'h, Pezennec, & Famelart,  
87 2011).

88 During milk acidification - carried out either with glucono delta lactone (GDL) or with lactic acid  
89 bacteria - the pH gradually decreases. This leads to physicochemical modifications of the casein  
90 micelles through, for instance, i) the progressive cancellation of negative charges of caseins due  
91 to the protonation of carboxylic acid functions and ii) the solubilization of minerals, such as  
92 colloidal calcium phosphate, that were stabilizing the micellar structure (Walstra, 1990). When  
93 the pH reaches 4.6, the electrostatic repulsions are cancelled and hydrophobic and hydrogen  
94 interactions become preponderant, allowing the caseins to form a network. If their concentration  
95 is large enough, a firm gel is finally formed (Anema & Li, 2000; Famelart, Lapesant, Gaucheron,  
96 Le Graet, & Schuck, 1996; Le Graet & Brulé, 1993; Lucey & Singh, 1997; Vasbinder et al.,  
97 2001).

98 Due to denaturation and aggregation, different types of aggregates can be produced when heating  
99 a whey protein solution, depending on the pH of the solution, the ionic strength and the protein  
100 concentration (Jung, Savin, Pouzot, Schmitt, & Mezzenga, 2008; Nicolai, Britten, & Schmitt,  
101 2011). When the pH reaches the isoelectric point, the proteins are weakly charged and will easily  
102 aggregate in order to form dense and spherical aggregates called microgels (Donato, Schmitt,  
103 Bovetto, & Rouvet, 2009). Microgel aggregates can also be obtained at a pH close to neutral in  
104 the presence of calcium (Phan-Xuan et al., 2014). When the pH is close to 7, whey proteins are  
105 negatively charged and fractal aggregates can be obtained by heat treatment (Nicolai, Britten, &  
106 Schmitt, 2011). Under this pH condition, the addition of NaCl leads to a decrease of the  
107 electrostatic repulsions. This results in the formation of large and dense fractal aggregates  
108 (Mahmoudi, Mehalebi, Nicolai, Durand, & Riaublanc, 2007). A final type of aggregates can be  
109 obtained by heating a WP solution in the presence of casein micelles at a pH of 6.3. In this case,  
110 the denatured whey proteins are known to bind to the micelle surface to form what is generally  
111 called mixed aggregates. The fine ratio of caseins and whey proteins contained in these mixed  
112 aggregates have been determined in former studies (Anema & Li, 2000, 2003; Loiseleux et al.,  
113 2018). Whereas the impact of native whey proteins on gel firmness has been extensively studied,  
114 it has not been clearly defined how pre-aggregates of different morphologies affect the formation  
115 of gel. The nature of protein aggregate is supposed to influence the interactions with the network  
116 of casein or to interact with the interface of fat droplet. This should modulate the rheological  
117 properties of acid milk gels. Torres et al. (2018) already demonstrated that a high content of  
118 native whey proteins associated with microparticulated whey proteins considerably increased the

119 viscosity, elasticity and resistance of stirred yoghurts. Microparticles behave like active fillers in  
120 the network of proteins and decrease whey separation.

121 The texture of an acid gel prepared from milk is also related to its fat content and to the size of  
122 the fat droplets. Increasing the fat content increases the strength of acid milk gels and decreases  
123 the duration to form a gel (Aguilera et al., 1993). This has been shown to result from the  
124 interactions between caseins and the proteins present at the surface of fat droplets (Aguilera &  
125 Kinsella, 1991). Moreover, increasing the pressure for the homogenization decreases the size of  
126 fat droplets (Jost, Baechler, & Masson, 1986). Other parameters alter the size of fat droplets such  
127 as the concentration of emulsifiers and the nature of proteins (Dickinson, 2003). For example, as  
128 native whey proteins have a high ability to cover large surface, their addition to model o/w  
129 emulsions produces fat droplets with smaller size than those obtained when whey protein  
130 aggregates (WPA) are added (Kiokias & Bot, 2006).

131 Whey separation is a common defect in yoghurts. It can occur especially if the gel network is  
132 damaged or if the gel undergoes a major structural reorganization (Lucey & Singh, 1997).  
133 Spontaneous whey separation can be defined as shrinking of the gel without application of  
134 external forces. It is related to the instability of the gel network that cannot trap the entire liquid  
135 phase anymore. Whey separation can be reduced, for example, by increasing the total solid  
136 content. There are two ways of acting on this parameter: increasing fat or protein concentrations  
137 (Delikanli & Ozcan, 2017; Isleten & Karagul-Yuceer, 2006; Keogh & O’Kennedy, 1998).  
138 Adding increased amounts of proteins before acidification results in a decrease of porosity in the  
139 gel. The protein network becomes denser and the protein material is better distributed inside the  
140 gel. This improves the homogeneity of the gel structure (Andoyo, Guyomarc’h, Burel, &  
141 Famelart, 2015; Andoyo, Guyomarc’h, Cauty, & Famelart, 2014). The presence of fat leads to  
142 more connections between proteins in the continuous phase (residual native whey protein or  
143 soluble and insoluble aggregates) and proteins localized at the interface, leading to a decrease in  
144 whey separation (Keogh & O’Kennedy, 1998).

145 As shown before, the effect of adding whey protein in milk before acidification on the final gel  
146 properties is better known than the one of protein aggregates. The aim of this study is to  
147 determine how adding different WPA can alter the rheological properties of acid milk gels (with  
148 or without fat in their content), and how WPA reduce whey separation. Different concentrations

149 and types of protein aggregates were studied, together with different homogenization pressures  
150 and fat contents.

## 151 **2- Materials and methods**

### 152 **2.1- Raw materials**

153 Anhydrous milk fat TREX 32C (melting point 32 °C) was kindly provided by Corman  
154 (Limbourg, Belgium). Whey protein isolate (WPI), casein powder (Cas), milk permeate powder  
155 and milk powder were supplied by local dairy companies (confidential origin).

156 Low heat WPI powder was used as the source of purified whey proteins. It was obtained as  
157 described by Chevallier et al. (2018). Briefly, milk microfiltrated by ultrafiltration and  
158 diafiltration was used to isolate a whey protein concentrate. The concentrate was then spray  
159 dried. This powder contained 91.1% (w/w) proteins - determined by the Kjeldahl method -,  
160 5.21% moisture,  $\leq 3\%$  lactose,  $< 3\%$  mineral and  $\leq 0.4\%$  fat. The protein content of the WPI  
161 powder was composed of 12% of caseins and 88% of whey proteins. Cas was composed of a  
162 small fraction of whey proteins (6.6%) and 81.7% (w/w) of caseins which were mostly in a  
163 micellar state (Loiseleux et al., 2018). This powder contained  $\leq 6\%$  moisture, 4% lactose, 4%  
164 mineral and  $\leq 2\%$  fat. Milk permeate powder was produced through ultrafiltration of skimmed  
165 milk using a membrane (molecular weight cut-off of 10 kDa) and was used in this study as a  
166 source of milk-soluble minerals. This powder contained  $< 4\%$  moisture,  $> 80\%$  lactose, 4%  
167 protein and  $\leq 1\%$  fat. The mineral content of the milk permeate powder determined by atomic  
168 adsorption was composed as following:  $\text{Ca}^{2+}$ : 0.31%;  $\text{Mg}^{2+}$ : 0.12%;  $\text{Na}^{+}$ : 0.63%;  $\text{K}^{+}$ : 2.74%. The  
169 milk powder was obtained from a skimmed milk obtained by atomization and having undergone  
170 a minimum heat treatment. The casein/whey protein ratio, given by the supplier, was close to  
171 80/20. The composition of this milk powder is given in Table 1.

### 172 **2.2- Production of aggregates and recombined milk preparation**

173 The protein aggregates (fractal, microgel and mixed aggregates) were produced according to the  
174 protocols used by Loiseleux et al. (2018) which are briefly summarized below. Once produced,  
175 the aggregates were cooled down at room temperature and stored at 4 °C for a maximum of 1  
176 month. To avoid microbial development, sodium azide at 0.2 g.L<sup>-1</sup> was added to all heated

177 solutions. It was confirmed by dynamic light scattering measurements that aggregate  
178 morphologies did not vary during storage (see 2.3.1).

#### 179 2.2.1- Fractal aggregates

180 WPI powder was dissolved overnight under magnetic stirring at 4 °C in milli-Q water (200 mL)  
181 at a concentration of approximately 80 g.L<sup>-1</sup>. Protein concentration was checked using a UV  
182 1800-spectrophotometer (Shimadzu, Kyoto, Japan) by absorption measurement at 278 nm using  
183 an extinction coefficient of 1.046 L.mol<sup>-1</sup>.cm<sup>-1</sup> (Mahmoudi et al., 2007). The solution was diluted  
184 to 50 g.L<sup>-1</sup> using milli-Q water. NaCl (final concentration: 0 to 45 mM) was added in order to  
185 obtain different sizes of fractal aggregates. The pH was adjusted to 7 with sodium hydroxide. A  
186 volume of 200 mL was subsequently heated at 80 °C for 2 h without stirring in order to produce  
187 fractal aggregates.

#### 188 2.2.2- Microgel aggregates

189 The WPI solution was prepared at 50 g.L<sup>-1</sup> as previously described. The pH was adjusted to 5.8  
190 with hydrochloric acid. A volume of 200 mL was heated at 85 °C for 1 h without stirring in order  
191 to produce microgel aggregates.

#### 192 2.2.3- Mixed aggregates

193 To be close to the mineral composition of milk and maintain the integrity of the casein micelles,  
194 an aqueous solution of milk permeate was used as a solvent. On the one hand, 100 mL of a 50  
195 g.L<sup>-1</sup> solution of whey proteins (WP) were prepared in a 56 g.L<sup>-1</sup> aqueous milk permeate solution  
196 and were left under stirring overnight at 4 °C. On the other hand, 200 mL of a 50 g.L<sup>-1</sup> casein  
197 dispersion (Cas) were also prepared in the same milk permeate solution under stirring at 20 °C  
198 for 30 min. Hydrochloric acid (6 M) was added to the Cas dispersion in order to reach a pH of  
199 6.3. The dispersion was maintained under magnetic stirring during 45 min at 60 °C, and then  
200 overnight at 20 °C to correctly rehydrate the powder. The WP solution and the Cas dispersion  
201 were mixed in order to obtain a 80/20 Cas/WP ratio (matching the milk ratio) at a total protein  
202 concentration of 50 g.L<sup>-1</sup>. The pH of the mix was adjusted to 6.3 with hydrochloric acid (6 M)  
203 and the solution was heated at 80 °C for 1 h without stirring.

#### 204 2.2.4- Preparation of recombined milk

205 The milk was reconstituted by reincorporating skimmed milk powder in a volume of Milli-Q  
206 water under magnetic stirring (500 rpm for 1.5 h) at room temperature to obtain a final protein  
207 concentration of 35 g.L<sup>-1</sup>. 2 to 15 g.L<sup>-1</sup> of native whey proteins or protein aggregates were added  
208 to the milk with a stabilization time of 15 min. The recombined milk that did not contain WP or  
209 protein aggregates was named “control milk”.

210 Systems with or without fat were studied. In the first case, anhydrous milk fat (AMF) and milk  
211 were heated at 60 °C before mixing. Homogenization was then carried out on the mixture (3.5%  
212 or 10% AMF) in two consecutive stages. A volume of 300 mL of sample was pre-emulsificated  
213 with a rotor-stator homogenizer SilentCrusher M (Heidolph, Germany) for 3 min at 14,000 rpm  
214 with a 12 mm head. Then, a high-pressure homogenizer Panda plus 1000 (GEA Niro Soavi, Italy)  
215 was used to reduce the size of the fat droplets, through recirculation at 100 or 500 bars at 29  
216 L/min for 5 min (with an average of 6 cycles). 50 mL of sample were heated under magnetic  
217 stirring at 90 °C for 11 min (including the temperature rise). It was then cooled down with ice for  
218 15 min, and stirred at 40 °C for 5 min. The residual NWP content was determined after the heat  
219 treatment of recombined milk. It was of 6 g/L and this value was constant for all the samples.  
220 Glucono- $\delta$ -lactone (GDL) at a pre-determined concentration, depending on the protein and the fat  
221 contents, was added to the systems in order to acidify them at a pH value of about 4.6 after 6 h  
222 (1.1% -1.3% (w/w)). The experimental steps of recombined milk preparation with fat are  
223 presented in Figure 1.

224 In absence of fat, the heat treatment was directly performed on 50 mL of sample after adding  
225 proteins to the reconstituted milk. A GDL concentration within the same range as for the  
226 recombined milk with fat was added.

## 227 **2.3- Aggregate characterization**

### 228 2.3.1- Size distribution of aggregates

229 A Zetasizer Nano Series (Malvern Instrument, UK) was used to determine the size (Z-average  
230 hydrodynamic diameter) and the size distribution of the aggregates after the heat treatment with  
231 dynamic light scattering (DLS). The aggregate solutions were diluted 100 times with Milli-Q  
232 water. The diluted solutions were placed in a plastic cell and analyzed at 20 °C in a  
233 backscattering configuration at 173° for 120 s. The hydrodynamic diameter was measured in

234 triplicate. A refractive index of 1.45 was used for protein particles (Zhang, Arrighi, Campbell,  
235 Lonchamp, & Euston, 2016) in order to calculate the volume-size distribution.

### 236 2.3.2- Transmission Electron Microscopy (TEM)

237 Aggregate solutions were diluted 500 times in Milli-Q water. A volume of 15  $\mu$ L of diluted  
238 sample was deposited on a carbon grid (200 meshes). After 2 min, the excess was removed with  
239 an absorbent paper. The samples were stained with 2% of uranyl acetate solution for 2 min before  
240 removing the excess, and then dried for 15 min at 60 °C. The aggregates were observed using a  
241 transmission electron microscope JEM-1230 (JEOL, Tokyo, Japan) operating at 80 kV.

## 242 **2.4- Analyzes of milk emulsions**

### 243 2.4.1- Fat droplet size determination

244 The fat droplet size distribution in emulsions was determined directly after emulsification using a  
245 laser light scattering instrument (Partica LA-960, Horiba, Kyoto, Japan), and at least one hour  
246 after a tenfold dilution by 1 % (w/v) of sodium dodecyl sulfate (SDS), in order to respectively  
247 obtain the size of the flocs and of the individual droplets. Size distribution was estimated using  
248 the Mie theory (Mie, 1908) and a refractive index of 1.458 for the dispersed phase (anhydrous  
249 milk fat) and 1.333 for the continuous phase (water). The mean diameter of the fat droplets was  
250 evaluated by the volume average diameter ( $D_{43}$ ).

### 251 2.4.2- Determination of the interfacial composition

252 Proteins were recovered from the fat droplet surface using the Patton & Huston (1986) method  
253 modified by Surel et al. (2014). The studied systems contained 3.5% or 10% (w/w) of AMF (to  
254 increase the interfacial surface) and 1% (w/w) of WP or aggregates. This analyze was performed  
255 on the milk after the homogenization step at 100 bars and after the heat treatment, but before  
256 gelation. This technique makes possible to separate the fat droplets from the emulsions in a single  
257 centrifugation step. 2 mL of emulsion were diluted in 2 mL of 50% (w/v) saccharose solution in  
258 order to increase the density of the aqueous phase. It was then slowly deposited at the bottom of a  
259 15 mL-centrifuge tube under 10 mL of a 5% (w/v) saccharose solution. The tube was then  
260 subjected to moderate centrifugation (3,000 g for 2 h at 20 °C) which caused creaming of the  
261 droplets. The droplets passed through the 5% (w/v) saccharose solution and were washed from all

262 unabsorbed constituents. After centrifugation, three phases were obtained: the top one (1) with  
263 the cream containing the washed fat droplets, the intermediate phase (2) with the 5% (w/v)  
264 saccharose solution, and the bottom one (3) with the aqueous phase of the emulsion containing  
265 the unabsorbed constituents.

266 The tubes were then immediately frozen at -20 °C in a vertical position to avoid redispersion of  
267 the different phases. They were then cut to recover each phase separately and study their  
268 composition. A prior desorption of proteins adsorbed at the interface was necessary. The top part  
269 (1) was recovered, weighed and diluted to 1/3 in a solution of 1% SDS. The mixture was  
270 vortexed and left standing for 1 h at 40 °C. It was then centrifuged at 3,000 g for 1 h at 10 °C.  
271 Two phases were obtained, the top phase (4) containing fat droplets stabilized by SDS and the  
272 bottom phase (5) containing the solution of desorbed proteins. The bottom phase (5) was  
273 recovered using a syringe, then diluted in a denaturing solution containing 8 M of urea, 5 g/L of  
274 SDS, 0.2 M of tris buffer, 0.05 M of trisodium citrate and 10 mM of  $\beta$ -mercaptoethanol ( $\beta$ Me).  
275 The aqueous phase (3) after thawing was also diluted in this denaturing solution. For phases 3  
276 and 5, the protein concentration was quantified by UV absorption at 278 nm using a 1800-  
277 spectrophotometer (Shimadzu, Kyoto, Japan) for which extinction coefficient was set up to 1.046  
278 L.mol<sup>-1</sup>.cm<sup>-1</sup> (Mahmoudi et al., 2007). The protein composition of the milk and of phases 3 and 5  
279 was determined by SDS-PAGE.

#### 280 2.4.3- Protein composition

281 The proteins adsorbed onto the fat droplet surface of the milk (phase 5), with or without added  
282 whey proteins or aggregates, were identified by SDS-PAGE. The solubilization buffer was  
283 prepared in a reducing condition (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10%  $\beta$ Me,  
284 0.1% bromophenol blue) or non-reducing condition (same composition without  $\beta$ Me). Phases 3  
285 and 5 were diluted to a concentration of 2 mg.mL<sup>-1</sup> and a volume of this solution was again  
286 diluted in one volume of solubilization buffer. The samples were then placed for 5 min at 100 °C  
287 in order to denature the proteins.

288 SDS-PAGE were performed using 10-well precast gels with a polyacrylamide concentration  
289 ranging from 4 to 12% (Bolt™ 4-12% Bis-Tris Plus, Invitrogen, Thermo Fisher Scientific,  
290 Waltham, United-States). In order to estimate the molecular weight of the proteins, a molecular

291 standard (Novex® Sharp Pre-Stained Protein Standard, Invitrogen, Thermo Fisher Scientific,  
292 Waltham, United States of America) was deposited in the first well. In each well, 10 µL of  
293 standard or 15 µL of sample were deposited. The gels were placed in the migration vessel  
294 containing the MES-SDS 1X (Bolt MES SDS running buffer (20X), Novex, Life Technology,  
295 Carlsbad, United States of America) and electrophoresis was carried out at constant current (60  
296 mA) for 1.5 h with a power source generator at 300 V (VWR, Radnor, United States of America).  
297 The gels were stained with Instant Blue™ (Expedeon, Cambridgeshire, United Kingdom) and  
298 scanned to identify proteins.

## 299 **2.5- Acid gelation characterization**

### 300 2.5.1- Rheological measurements of acid milk gels

301 Rheological experiments were performed using a AR 2000 controlled stress rheometer (TA  
302 Instruments, Leatherhead, United Kingdom) equipped with a Couette geometry (stator radius: 15  
303 mm; rotor radius: 14 mm; cylinder immersed height: 42 mm) thermostated by a Peltier module.  
304 The sample was deposited at 40 °C in the outer cylinder just after the addition of GDL ( $t_0$ ). The  
305 inner cylinder was lowered until it was completely immersed in the sample, and then the pH  
306 electrode (Mettler Toledo InLab® Micro, Mettler Toledo, Schwerzenbach, Switzerland) was  
307 placed in the sample above the Couette geometry for *in situ* measurements. The sample was then  
308 covered with paraffin oil to prevent evaporation.

309 A time sweep was first done to follow the changes in  $G'$  and  $G''$  during 6 h of acidification (1  
310 rad/s, strain 1%) at 40 °C while the pH was measured. When the pH reached 4.6, a frequency  
311 sweep (0.01 to 100 rad/s, strain 1%) was performed at a constant temperature of 40 °C, followed  
312 by a strain sweep (0.02 to 4%, 1 rad/s, 40 °C) to ensure that the measurements were done in the  
313 linear viscoelastic region. The measurements were performed at least twice. Time and pH of  
314 gelation, as well as final  $G'$  were determined from the acidification kinetics. The change of loss  
315 tangent ( $\tan \delta = G''/G'$ ) versus time and pH were analyzed in order to obtain the gelation time  
316 and pH from the asymptote of  $\tan \delta$  (Lucey, Munro, et al., 1998; Lucey, Teo, et al., 1998). The  
317 amplitude of the loss tangent at gelation pH was also determined. The  $G'$  slope was determined  
318 from the frequency sweep and gave information on the structure and the strength of the gels  
319 formed at pH 4.6.

### 320 2.5.2- Confocal Laser Scanning Microscopy (CLSM)

321 A NIKON Eclipse-TE2000-A1si (Champigny sur Marne, France) confocal laser scanning  
322 microscope was used to characterize the microstructure of gels containing 3.5 % (w/w) of fat and  
323 1 % (w/w) of WP or aggregates. Immediately after the GDL addition, a droplet of milk sample  
324 was deposited on a microscope slide equipped with a spacer that has a depth of 250 µm. Several  
325 microliters of two dyes, Nile Red (for lipids) dissolved in dimethylformamide (0.5 µg/µL) and  
326 Alexa 488 (for proteins) dissolved in methanol (0.2 µg/µL), were added to the sample and mixed.  
327 The system was sealed with a cover glass to prevent evaporation. The samples were covered with  
328 aluminum foil - to protect the fluorescent probes from light until sample analysis - and placed in  
329 an oven maintained at 40 °C for 6 h. Samples with a pH of 4.6 were observed using a x20 lens  
330 (Plan APO 20X; numerical aperture: 0.75) and a water immersion lens x40 (Plan APO 40X;  
331 numerical aperture: 1.25) using an Argon laser (corresponding to excitation of the Alexa 488) and  
332 the 561 nm Laser Diode (Nile Red).

### 333 2.5.3- Whey separation analysis

334 Whey separation was determined according to the centrifugation method (Keogh & O’Kennedy,  
335 1998) with some modifications. Immediately after stirring, 10 mL of sample with GDL were  
336 placed in three previously weighed tube and stored at 40 °C for 6 h, and then at 4 °C for one night  
337 (D1), 7 days (D7) or 14 days (D14). Centrifugation at 4 °C and 3,500 rpm for 15 min were  
338 performed at D1, D7 and D14. After 1 min of incubation without stirring, the supernatant was  
339 recovered and weighed. Whey separation was calculated according to the following equation  
340 (Keogh & O’Kennedy, 1998):

$$341 \quad \text{Whey separation (\%)} = (\text{Weight of supernatant (g)} / \text{Weight of gel sample (g)}) \times 100$$

### 342 **2.6- Statistical analyses**

343 The presented results were obtained from at least two repetitions of complete sample preparation  
344 and analysis. ANOVA was performed using StatGraphics ® Plus, 5.1 (Statpoint Technologies,  
345 Inc., Warrenton, VA, USA) with a Tukey test (95.0%). Statistical analyses were performed on  
346 rheological analysis on control acid milk gels with or without fat, on acid milk gels with 1%  
347 added WP, and fractal, microgel or mixed aggregates with or without fat. These statistical

348 analyses were also performed when studying whey separation, the effect of the size of fractal  
349 aggregates and the one of homogenization pressure.

### 350 **3- Results and discussion**

#### 351 **3.1- Aggregate characterization**

##### 352 3.1.1- Fractal aggregates

353 The Z-average diameter of fractal aggregates produced at 80 °C during 2 h at 45, 20 and 0 mM of  
354 added sodium chloride was respectively 228, 93 nm and 68 nm (Figure 2A). Depending on their  
355 size, the fractal aggregates were called Fa228, Fa93 and Fa68. Observations made by TEM  
356 evidenced a more branched structure for Fa228 than the ones produced at the lowest NaCl  
357 content (Fa93 and Fa68) (Figure 2B). This reflects a higher size polydispersity for the aggregates  
358 present in Fa228 by comparison to those present in Fa93 and Fa68. TEM observations show  
359 Fa228 fractal aggregates forms cluster and over-aggregates. Consequently, the structures and the  
360 size of fractal aggregates could be monitored according to the ionic strength of the solution.

##### 361 3.1.2- Microgel aggregates

362 Microgel aggregates exhibited a Z-average diameter of 450 nm (Figure 3A). They appeared in the  
363 TEM as dense, circular and were mostly grouped (Figure 3B). These observations explain the  
364 results obtained by dynamic light scattering (DLS) measurement. Individual microgel aggregates  
365 exhibited a diameter of 150-200 nm.

##### 366 3.1.3- Mixed aggregates

367 The size distribution of mixed aggregates could not be obtained using DLS, because it was not  
368 possible to discriminate between casein micelles and mixed aggregates (Loiseleux et al., 2018).  
369 Mixed aggregates observed by TEM (Figure 4) exhibited, as expected, a core composed of a  
370 casein micelle (average diameter between 100 and 200 nm) and a surface of whey protein  
371 aggregates that were more or less branched.

#### 372 **3.2- Gelation properties of acid milk gels with or without fat and protein aggregates**

##### 373 3.2.1- Control acid milk gels with or without anhydrous milk fat (AMF)

374 First experiments were made on control acid milk gels, namely without further addition of  
375 proteins, with or without 3.5% of AMF. For systems with AMF, homogenization at 100 bars  
376 resulted in fat droplets of a mean diameter of 0.8  $\mu\text{m}$  ( $D_{43}$ ).

377 The average results obtained for acid milk gels are presented in Table 2. Similar time (around 29  
378 min) and pH of gelation (5.45) were obtained independently of the presence of fat. However,  
379 compared to control acid gel without fat, fatty gels had a higher final storage modulus from over  
380 100 Pa. The  $G'$  slope obtained from the frequency sweep of the AMF gels was lower (0.150) than  
381 without AMF (0.164). This showed that the addition of anhydrous milk fat allowed the formation  
382 of stronger and more structured systems, in accordance with the results obtained by Aguilera et  
383 al. (1993).

#### 384 3.2.2- Impact of native whey protein addition on acid milk gel properties

385 In the same way, the gelation of acidified milk with or without fat in the presence of variable  
386 concentrations of native whey proteins added before heat treatment was also monitored. A linear  
387 increase of final  $G'$  with native whey protein (NWP) concentration added was obtained with or  
388 without fat. The values obtained for acid milk gels with AMF were of course higher (Figure 5A).  
389 Furthermore, for acid gels containing 1% added NWP, a strong decrease in gelation time and  
390 therefore an increase in gelation pH were obtained (Table 2). It has already been established that  
391 increasing whey protein concentration in milk before heat treatment induces a better structuring  
392 and higher firmness of acid gels, and a decrease of the gelation time (Lucey & Singh, 1997). The  
393 heat treatment applied on the milk induces the denaturation of whey proteins (Anema & Li, 2015;  
394 Cayot & Lorient, 1998; Chen & Dickinson, 1998; Paulsson & Dejmeek, 1990) and their  
395 interactions with each other or with  $\kappa$ -casein at the surface of the casein micelles. Whey protein  
396 aggregates formed when heating the gel at a pH of about 5.6, while pure casein micelles formed a  
397 gel of a pH of approximately 5.0 (Vasbinder, de Velde, & de Kruif, 2004). The higher gelation  
398 pH of whey protein aggregates can be explained by their pI values and also by their significantly  
399 higher surface hydrophobicity (Morand, Dekkari, Guyomarc'h, & Famelart, 2012; Morand,  
400 Guyomarc'h, Legland, & Famelart, 2012). During the acidification process, the WP aggregates  
401 were shown to interact with casein micelles and accelerate their destabilization. It causes both a  
402 decrease in gelation time and an increase in gel firmness (Andoyo et al., 2015; Ozcan, Horne, &  
403 Lucey, 2015).

404 Furthermore, compared to the control, a decrease of  $\tan \delta$  amplitude and  $G'$  slope from the  
405 frequency sweep was also obtained with 1% of WP regardless of the presence of AMF (Table 2).  
406 All these observations confirmed that a more structured protein network, with more connections  
407 or stronger bonds, was produced in the presence of a higher WP concentration. Lucey & Singh  
408 (1997) and van Vliet, van Dijk, Zoon, & Walstra (1991) considered that the  $\tan \delta$  amplitude is  
409 related to the possible relaxation of bonds during the initial period after the formation of gel. Less  
410 relaxation (lower  $\tan \delta$  amplitude) can be explained by a more solid-like system. It is noteworthy  
411 to mention that similar changes in  $G'$  were obtained with or without AMF. In both cases,  $G'$   
412 increased along with the NWP concentration. This can be seen in Figure 5A where the two lines  
413 are nearly parallel. By comparison with gel without AMF, the final  $G'$  of all gels containing  
414 AMF were higher. The increase of  $G'$  may evidence that fat concentrates proteins in the  
415 continuous phase by steric hindrance and that the interaction between the network of casein and  
416 the proteins coated at the interface of fat droplets has been improved. Increasing the fat  
417 concentration thus increases the strength of acid milk gels and shortens the gelation time  
418 (Aguilera & Kinsella, 1988, 1991; Aguilera et al., 1993). Fat droplets covered with proteins can  
419 then act as a nuclei on which native or aggregated whey proteins and casein micelles connect  
420 during gel formation (Jost, Dannenberg, & Rosset, 1989). In parallel, Aguilera & Kinsella (1988,  
421 1991) have also shown that the addition of WP to milk with fat improved the rheological  
422 properties of acid gels. In our case, the changes observed upon addition of NWP were similar  
423 regardless of the presence of AMF. It indicates that whey proteins mostly play a role in the main  
424 protein network and are weakly or not involved in the interactions at the interface. The presence  
425 of fat thus reinforces the protein network mainly by steric exclusion.

426 The results obtained for the separation of whey in fat systems are presented in Figure 5B.  
427 Separation of whey close to 0% was obtained for acid gels at 0.4 to 1% WP and was stable over  
428 time, while systems with no addition of whey protein showed a large separation of whey  
429 (between 10 and 15%). By comparison, the value obtained for systems without fat at 1% WP  
430 added was around 50%. This result is in agreement with the literature, Keogh & O'Kennedy  
431 (1998) having shown that fat decreased whey separation. The presence of fat together with a  
432 higher concentration of proteins increased the ability of the reinforced protein network to  
433 immobilize the aqueous phase. Furthermore, it has also been established that increasing protein  
434 concentration reduced whey separation (Delikanli & Ozcan, 2017; Isleten & Karagul-Yuceer,

435 2006), and nonfat yogurts fortified with WPI had a lower level of whey separation than without  
436 addition of WPI (Isleten & Karagul-Yuceer, 2006).

### 437 3.2.3- Impact of protein aggregate addition on acid milk gelation

438 Acid milk gels with or without 3.5% AMF and with 0.2 to 1% of fractal aggregates (Fa228) as  
439 well as 0.6 to 1.5% of microgels or mixed aggregates were produced. For microgel and mixed  
440 aggregates, the lowest concentrations (0.2 and 0.4%) were not tested, as analyses on systems  
441 without fat did not show any changes in the final  $G'$  and whey separation compared to control  
442 acid milk gels. The results for acid gelation of 3.5% AMF systems containing protein aggregates  
443 are reported in Figure 6 and Table 2.

444 In presence of 1% Fa228, a decrease in gelation time related to an increase in gelation pH was  
445 observed with or without AMF compared to the control, as it is the case in systems with WP  
446 (Table 2). Furthermore, the addition of increasing amounts of fractal aggregates also led to a  
447 linear increase of  $G'$  (Figure 6). Additionally, as it was the case with acid gels without fat, the  $G'$   
448 values were lower than those obtained when adding NWP. Addition of these fractal aggregates  
449 also led to both a lower  $G'$  slope and  $\tan \delta$  value. Although  $G'$  was lower, indicating less  
450 connections, these two last parameters indicated a better structuration of the gel, probably  
451 because of stronger interactions between the fractal aggregates and the casein network. The whey  
452 separation analysis showed the same changes than when adding NWP, in the presence and the  
453 absence of added fat (Figure 7). Whey proteins and fractal aggregates were both able to  
454 participate in the formation and the strengthening of the protein network, without strong  
455 interactions with the fat droplet interface.

456 By contrast, as shown in Table 2, adding microgel aggregates (1%) did not affect the final  $G'$   
457 value of acid milk gels with or without AMF compared to the control with or without AMF.  
458 Furthermore, as shown in Figure 6, the final storage moduli after 6 h of acidification were stable  
459 for gels with AMF and 0.6 to 1.5% of microgel aggregates. However, the rheological analyses  
460 showed a significant increase in gelation time and a decrease in gelation pH compared to controls  
461 (shown for a concentration of 1% in Table 2). This means that, by contrast with the addition of  
462 WP and fractal aggregates, preformed microgels did not establish interactions with any  
463 component of the system and were unable to strengthen the texture. It may be due to their larger

464 size and higher density compared to fractal structures. Microgel aggregates were less numerous  
465 than WP or fractal aggregates and they seemed to have an inert role in the network. These results  
466 are in agreement with those obtained by Liu et al. (2016), who have shown that aggregates of  
467 smaller sizes were more effective for texturing acid milk gels without fat. They explained that  
468 microparticulated aggregates, unlike nanoparticulate aggregates, did not form strong structures  
469 via disulphide interactions with proteins in milk without fat during heat treatment and were also  
470 unable to become part of the protein network. During acidification, larger aggregates may restrict  
471 bonding with both caseins and other whey proteins, so the protein network was less structured.  
472 These results were different from those reported by Torres et al. (2018), showing an increase of  
473 the texture of yoghurts upon addition of such aggregates. This was ascribed to an active filling of  
474 the aggregates, establishing connections with the main protein network. The authors pointed out  
475 that those residual native whey proteins were included in their aggregate samples, explaining  
476 their higher functional effect after heat treatment. In our case, very few NWP were present in the  
477 samples (Loiseleux et al., 2018) and increasing microgel aggregate amounts did not lead to  
478 change the rheological properties. Furthermore, the aggregates used in this study are produced at  
479 a higher temperature than the ones used by Torres et al. (2018). This parameter potentially was  
480 the origin of the differences in functionality. In addition, the whey separation obtained on acid  
481 milk gels with AMF and with addition of 1.5% of microgels (Figure 7) showed similar results  
482 compared to the control gels (between 10 and 15% of whey separation). This high level of whey  
483 separation confirms the assumption that these aggregates exhibit only an inert role, unlike fractal  
484 aggregates or WP. However, their incorporation makes it possible to increase the protein  
485 concentration in acid milk gels without significantly altering their firmness properties. This was  
486 observed independently of the presence of fat.

487 The same type of results was obtained in the presence of mixed aggregates. Their addition did not  
488 lead to an increase in the storage modulus of the acid milk gels with AMF (Figure 6).  
489 Furthermore, the rheological analyses showed a slight decrease in the gelation pH compared to  
490 the control gel (shown for a concentration of 1% in Table 2). But, in the contrary of what  
491 occurred in the presence of microgels, mixed aggregates triggered a decrease in whey separation  
492 compared to control. Nevertheless, this was less effective than fractal aggregates or WP (Figure  
493 7). They partially prevented the release of whey probably by decreasing the size of the gel pores  
494 or due to their water retention capacity. By using this type of aggregates, it became possible to

495 obtain acid milk gels with constant firmness and reduced whey separation even at the highest  
496 protein content. The consistency of gel modulus upon addition of mixed aggregates was quite  
497 surprising knowing that such aggregates are formed *in situ* via disulfide bridges during the heat  
498 treatment of conventional milk (Anema & Li, 2000; Haque & Kinsella, 1988; Jang & Swaisgood,  
499 1990). In this case, mixed aggregates together with fractal aggregates induced an increase in gel  
500 strength (Donato & Guyomarc'h, 2009; Mahmoudi et al., 2011, 2010; Vasbinder et al., 2001).  
501 The addition of preformed mixed aggregates did not increase gel strength. They were probably  
502 inert due to the heat treatment used to produce the aggregates. This means that they were stable  
503 structures with different functionalities than the aggregates formed *in situ*. Moreover, these  
504 aggregates were not able to create connections with the protein network that forms during  
505 acidification.

#### 506 3.2.4- Effect of the size of fractal aggregates and homogenization pressure at 3.5 and 10% AMF

507 The impact of different sizes of protein aggregates on the textural and microstructural properties  
508 of acidified milk model systems without fat has already been reported (Liu et al., 2016) and  
509 showed that smaller aggregates provide acid gels with higher firmness than larger aggregates. To  
510 evaluate the role of the interface or the one of the protein network on the strengthening of the  
511 acid dairy gels observed in the previous results, an increase in the interfacial surface was  
512 produced. This, in order to monitor if aggregates can modulate the texture of acid milk gels by an  
513 action at the interface.

514 Systems that contained 3.5 or 10% of anhydrous milk fat and fractal aggregates of different sizes  
515 were produced and homogenized at 100 or 500 bars before acidification. As previously stated, the  
516 mean diameter ( $D_{43}$ ) of fat droplets was of approximately 0.8  $\mu\text{m}$  in the systems containing 3.5%  
517 AMF that were homogenized at 100 bars. When these systems were homogenized at 500 bars, the  
518 mean diameter of fat droplets was of approximately 0.25  $\mu\text{m}$ . The step of homogenization also  
519 impacts the diameter of fat droplets in recombined milks containing 10% AMF. The mean  
520 diameter of the droplets was of approximately 0.9 and 0.3  $\mu\text{m}$  when the systems were  
521 homogenized at 100 and 500 bars, respectively. These conditions aimed to increase the total  
522 interfacial surface, to allow adsorption of other proteins than caseins at the interface. It has  
523 already been established that, with an identical fat concentration, emulsions containing small fat  
524 droplets reinforced the gels in a more pronounced way than emulsions with large fat droplets.

525 This reflected the importance of the number and sizes of the fat droplets in the gels (Xiong,  
526 Aguilera, & Kinsella, 1991).

527 The values of the final storage modulus obtained after 6 h of acidification in the presence of 1%  
528 fractal aggregates of different sizes are presented in Figure 8. Slightly higher values were  
529 obtained at 3.5% AMF and 100 bars in the presence of small fractal aggregates (Fa93 and Fa68).  
530 This result was also seen when recombined milk was homogenized at 500 bars, i.e. with a more  
531 developed interfacial surface. To further increase the interfacial surface, the same experiences  
532 were performed in the presence of 10% AMF. At 100 and 500 bars, the same trend was observed:  
533 an increased storage modulus was obtained when smaller fractal aggregates were used. This  
534 increase was more pronounced with a higher fat content (10%) and a high homogenization  
535 pressure, i.e. in the conditions where the interfacial surface was the highest. This change could be  
536 due to the development of higher number of interactions between the protein network and the  
537 aggregates because fractal aggregates of smaller size were in larger amount. The fact that no  
538 drastic change in the evolution of the moduli with the different aggregate was observed does not  
539 evidence a major role of the interface, except at 10% fat and 500 bars. The effect of decreasing  
540 aggregate sizes was mainly the same regardless of the fat content or the homogenization pressure.  
541 Under the tested conditions, this could mean that decreasing the size of the aggregates had mainly  
542 the same effect on the texture of gels, except in the case of the highest interfacial surface.

### 543 3.2.5- Confocal Scanning Laser Microscopy on acid milk gels

544 The microstructure of the acid gels containing 3.5% fat in the presence of 1% of various added  
545 proteins was observed (Figure 9) to visualize the protein network and the fat localization.

546 The control gel shows a sparse protein network (appearing in green), with large pores (in black).  
547 As a reminder, in this system, the rheological results gave a relatively weak elastic modulus and a  
548 significant whey separation was observed. The fat (in red) appeared well distributed within the  
549 gel and connected to the protein network.

550 The protein network in samples containing WP and fractal aggregates exhibited the same global  
551 aspect, but looked denser and more homogeneous than in the control. The pore sizes of the gels  
552 were seen decreased compared to control. This seems to support the higher results of elastic

553 modulus and the lower whey separation measured for these gels. The fat was well distributed  
554 within the gel and seemed connected to the protein network, as for the control. It has already been  
555 established that with a higher protein concentration, gel pores become smaller and a more  
556 homogeneous distribution of the protein material is observed. This leads therefore to a higher  
557 whey retention (Andoyo et al., 2014). The addition of whey protein had an impact on the  
558 establishment of the protein network and thus on the final texture of milk products. Fractal  
559 aggregates played the same role as WP. They seem encompassed in the protein network and  
560 contribute to its reinforcement.

561 The structure of the protein network containing microgel aggregates showed a greater  
562 heterogeneity than that of gels with WP or fractal aggregates, with proteins appearing more  
563 dispersed in the system. Larger whey zones compared to the gels with WP, quite similar to the  
564 structure of the control gel, were seen. This supports the rheological results which gave a final  
565 elastic modulus close to the value obtained for the control gel. Similar results were also obtained  
566 for whey separation. As the microgel aggregates were dense, they were less able to connect with  
567 the protein network.

568 The protein network with mixed aggregates also appeared visually more heterogeneous than  
569 when WP or fractal aggregates are added, but slightly more structured than with the addition of  
570 microgels. This evolution is the same than the one obtained for the whey separation results.  
571 Mixed aggregates appeared to be able to play a role, probably as inert filler, in the protein  
572 network, reducing the whey separation. The final elastic modulus obtained was close to the value  
573 of the control gel. The separate preparation of mixed aggregate and its further addition to milk  
574 did not lead to the same result as when they were formed *in situ*. This questions their structural  
575 difference in relation to their functionality. Even if it has been verified that the casein micelles  
576 used to manufacture the mixed aggregates were mostly in the micellar state, their fine structure  
577 could be different from the native one, hence impacting the functionality of the mixed aggregates.

578 These results are in agreement with those obtained by Liu et al. (2016). These authors have  
579 studied the impact of different types of protein aggregates of variable sizes on the textural and  
580 microstructural properties of acidified milk model systems without fat. The whey proteins were  
581 either nanoparticles ( $< 1 \mu\text{m}$ ) or microparticles (1 - 10  $\mu\text{m}$ ). Nanoparticulated WP provided acid  
582 gels with higher firmness, faster gelation and lower whey separation coupled with a denser

583 microstructure. In contrast, microparticulated WP only weakly interacted with other proteins and  
584 their addition resulted in a protein network with low connectivity. Thus, the nature of the  
585 aggregates does not seem to be the only parameter to be taken into account. Their size also seems  
586 to be a key parameter in the modulation of the rheological properties of acid dairy gels. In our  
587 case, in the presence of fat, the same results were obtained: an improved texture, lower whey  
588 separation and more structured protein networks were obtained with the whey proteins and with  
589 the smaller fractal aggregates. Texture modulation by the protein aggregates seemed then to be  
590 mostly obtained by the protein interactions in the continuous phase and not by the connection of  
591 proteins from the droplet interfaces.

### 592 **3.3- Interfacial composition**

593 Although we aimed to modify and control the texture of acid milk gels through the addition of  
594 protein aggregates by connecting fat droplets, the results described above lead to the assumption  
595 that the observed modulation of the texture could mainly be due to an involvement of these  
596 aggregates in the continuous phase and not on the interface. To confirm this hypothesis, the  
597 interfacial composition of fat droplets in the milk before acidification was analyzed by  
598 electrophoresis.

599 Control milk and milks containing WP, fractal (Fa68) or mixed aggregates were prepared. The  
600 milks were enriched with 10% AMF and homogenized at 100 bars, to increase the interfacial  
601 surface. Unfortunately, due to experimental reasons, it was not possible to study systems  
602 homogenized at 500 bars. Indeed, the size of the fat droplets in these systems was too small.  
603 Milks and different phases of milk (3: containing the unabsorbed constituents and 5: containing  
604 desorbed proteins from the fat droplets) according to the Patton process as described above (see  
605 2.4.2) were analyzed. Electrophoresis gels of milk and phase-5 and phase-3 control milks in non-  
606 reducing conditions, and phase-5 control milk and milks containing 1% WP, fractal or mixed  
607 aggregates in non-reducing and reducing conditions are presented in Figure 10.

608 The electrophoresis profile of total control milk in well call “milk” (Figure 10A) shows the  
609 presence of all the milk proteins. Bovine serum albumin (BSA), caseins,  $\beta$ -lactoglobulin and  $\alpha$ -  
610 lactalbumin were found. For the emulsion’s aqueous phase (3), all milk proteins were also  
611 present. The main proteins which were found were caseins, but  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin

612 were also present in this phase. In non-reducing conditions, protein aggregates that have not  
613 migrated were found at the top of the gel. These aggregates were found in the total control milk  
614 and aqueous phase (3) as well as in phase 5 which contained desorbed proteins from the fat  
615 droplets of the control milk. Compared to the other samples, phase 5 showed a less abundant  
616 content of protein aggregates and was mainly composed of caseins.

617 The aggregates found at the top of the electrophoresis gels were those that formed naturally  
618 during the heat treatment of milk. For systems containing added fractal and mixed aggregates  
619 (phase 5 in non-reducing condition), more aggregates at the top of the gel seemed to be found  
620 than for the control and system containing added WP. This would mean that some of the added  
621 preformed aggregates could have access to the interface of the fat droplets.

622 In phase 5, the electrophoresis profile in non-reducing conditions showed that these phases  
623 mostly contained caseins. Almost no  $\beta$ -lactoglobulin or  $\alpha$ -lactalbumin was found in all the tested  
624 systems. It has been established that caseins preferentially adsorb to the surface of fat droplets  
625 compared to WP (Srinivasan, Singh, & Munro, 1996; Ye, 2008). If the casein concentration is  
626 sufficient, caseins can completely cover the fat droplet surface (Chevallier et al., 2016, 2018).  
627 However, in all systems, a small amount of aggregates could access the interface. This was  
628 confirmed by analyses of phase 5 in reducing conditions where bands matching  $\beta$ -lactoglobulin  
629 and  $\alpha$ -lactalbumin were seen. These proteins were included in the composition of the aggregates  
630 present in the electrophoresis gels under non-reducing conditions and were probably also  
631 adsorbed on caseins. For phase 5 of milks with 1% fractal aggregates (Fa68) in reducing  
632 condition, more  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin were observed than in other systems. It shows  
633 that these aggregates may have easier access to the interface.

634 It is therefore reasonable to claim that the interface of fat droplets in milk with 10% AMF and  
635 homogenized at 100 bars, was mainly covered by milk caseins. The additional whey proteins,  
636 added in the native form or as aggregates, rather played a role in the continuous phase. However,  
637 aggregates could be found at the interface in very small amounts, and this was more pronounced  
638 in presence of fractal aggregates. The increase of fat concentration to 10% and a homogenization  
639 pressure of 100 bars were not sufficient to allow the aggregates to reach the interface in large  
640 numbers and cause a large modification of interface connectivity. It should be noted that the same

641 experiments were performed on control milk containing 3.5% AMF and milk with added fractal  
642 aggregates (results not shown). Almost no aggregates were found in the phase containing  
643 desorbed proteins from the fat droplets. This confirms the hypothesis made from the rheological  
644 results that the whey proteins or the aggregates were mainly involved in the formation of the  
645 protein network. At 3.5% AMF, no difference was observed in the interfacial composition  
646 between the control milk and the milk with added fractal aggregates. It means that the interface of  
647 fat droplets was completely saturated with caseins.

#### 648 **4- Conclusions**

649 This study has shown that adding different types of milk protein aggregates can change the  
650 texture of acid milk gels. The addition of whey proteins or fractal aggregates strengthened the  
651 structure of acid milk gels in the presence or in the absence of fat. Nevertheless, the addition of  
652 microgels or mixed aggregates had no impact on the rheological properties of acid milk gels.

653 The differences in textures obtained with the different types of aggregates with a low fat content  
654 (3.5%) were attributed to their involvement in the continuous phase of the systems. No  
655 significant impact of the aggregates on the composition of the fat droplet interface was  
656 evidenced, because of its saturation by the milk caseins. The different aggregates did not play the  
657 same role on the formation of the protein network: WP and fractal aggregates created a higher  
658 number of connections or stronger connections in the network which strengthened the protein  
659 network and reduced whey separation. Microgel and mixed aggregates behaved more like an  
660 inert material. Even if the acid dairy gel texture was not changed by adding such aggregates,  
661 mixed aggregates induced a decrease in whey separation. These types of aggregates allow for the  
662 formulation of protein-enriched acid dairy gels with controlled rheological properties.

663 The interfacial composition results showed that aggregates can access the interface when its  
664 surface is drastically increased. This limited access is due to the predominant ability of caseins to  
665 adsorb at interfaces. These results are in agreement with those found by Loiseleux (2017) who  
666 showed the ability of such aggregates to texturize emulsions containing 5% or 30% fat and very  
667 low concentrations of casein. Increasing fat content by over 10% or playing with the micellar  
668 caseins/WPN ratio or the fractal aggregates in milk acid gels could be a parameter to control the  
669 interfaces in such products and therefore could be used to modulate their texture. The difference

670 of functionality between preformed and *in situ* produced mixed and microgel aggregates should  
671 be further investigated.

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## Preparation of reconstituted milk (with AMF)

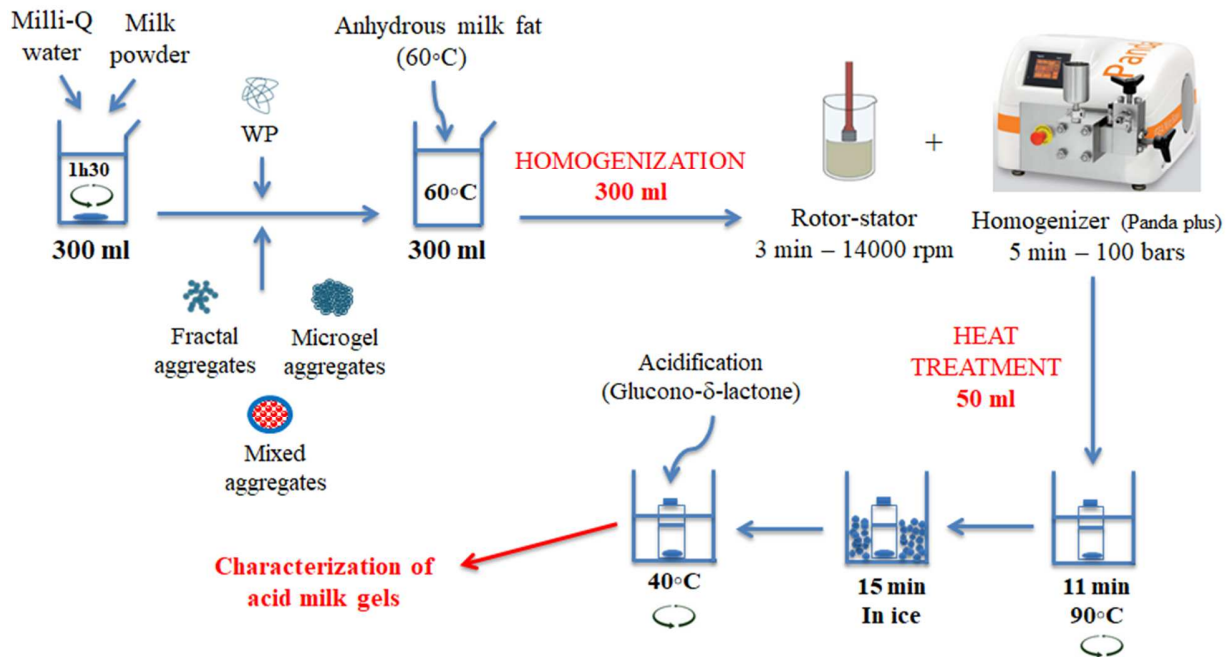


Figure 1: Experimental steps of preparation and acidification of reconstituted milk containing AMF and native whey proteins or protein aggregates

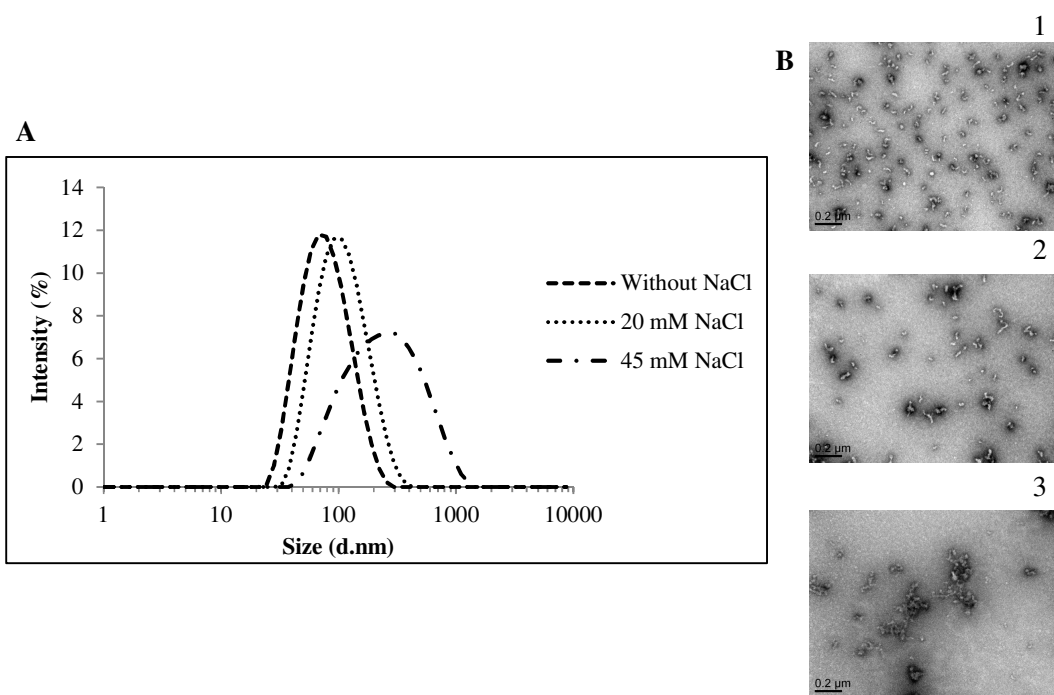


Figure 2: (A) Size distribution (DLS) of fractal aggregates obtained after heat treatment at 80 °C during 2 hours with variable concentrations of NaCl; (B) TEM micrographs (scale bar: 0.2  $\mu\text{m}$ ) of fractal aggregates stained with 2% uranyl acetate (1: 0 mM, 2: 20 mM and 3: 45 mM NaCl)

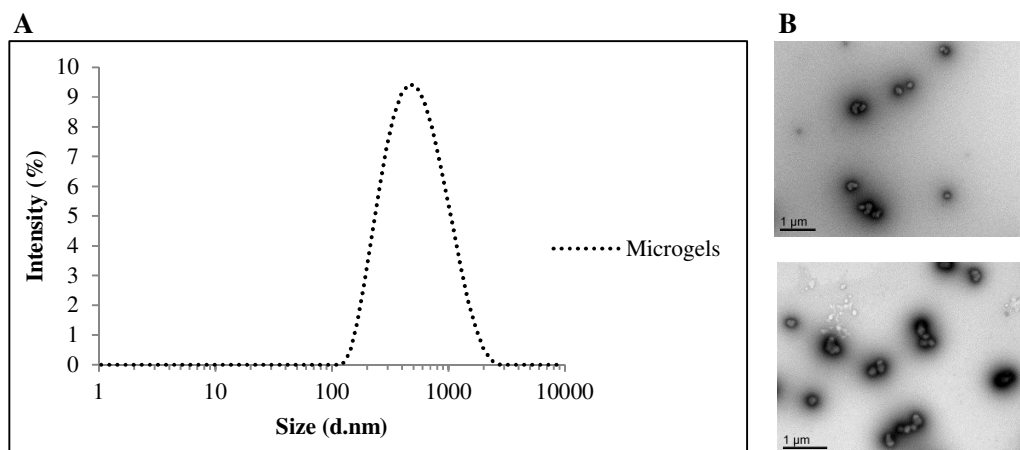


Figure 3: (A) Size distribution (DLS) of microgel aggregates obtained after heat treatment at 85 °C during 1 hour; (B) TEM micrographs of microgel aggregates stained with 2% uranyl acetate (scale bar: 1  $\mu\text{m}$ )

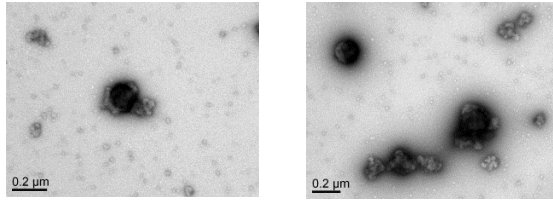


Figure 4: TEM micrographs of mixed aggregates obtained after heat treatment at 80 °C during 1 hour stained with 2% uranyl acetate (scale bar: 0.2 μm)

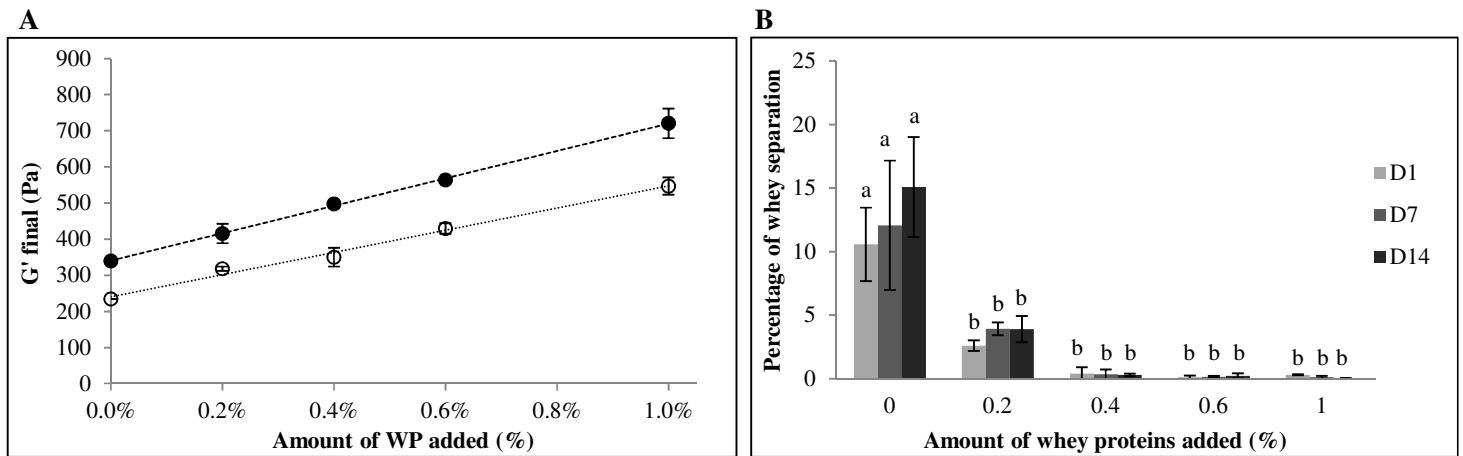


Figure 5: (A) Changes in the final storage modulus after 6 hours of acidification of acid milk gels with 3.5% AMF (black circle) or without AMF (empty circle) according to added WP quantity (0 to 1%); (B) Change of percentage of whey separation according to added WP quantity (0 to 1%) and time (day 1, 7 and 14) of gels at 3.5% AMF

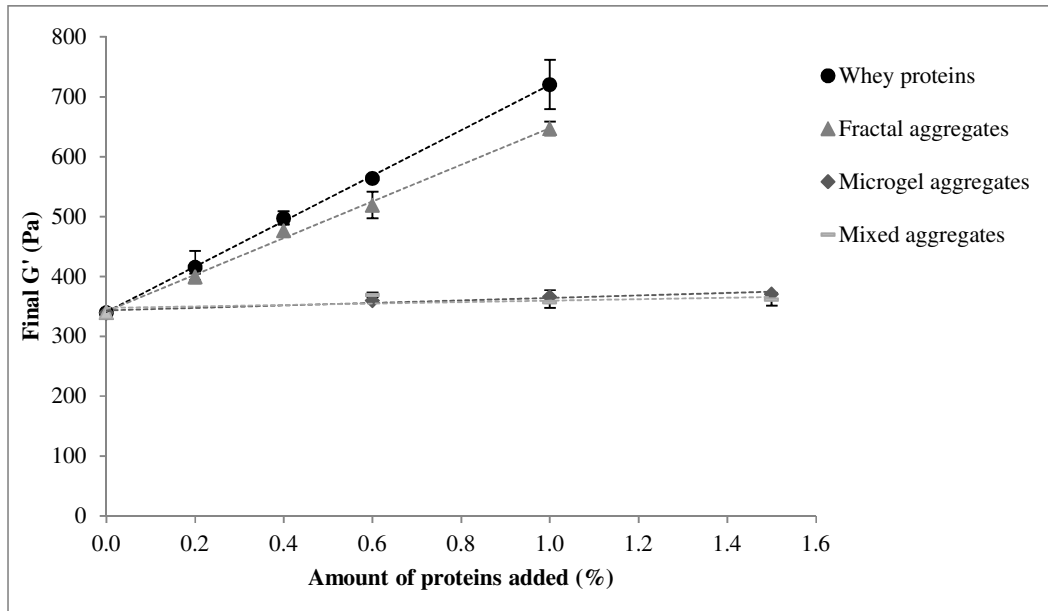


Figure 6: Change of the final storage moduli after 6 hours of acidification of acid milk gels (3.5% AMF) according to protein addition (whey proteins or fractal, microgel and mixed aggregates; 0 to 1.5%)

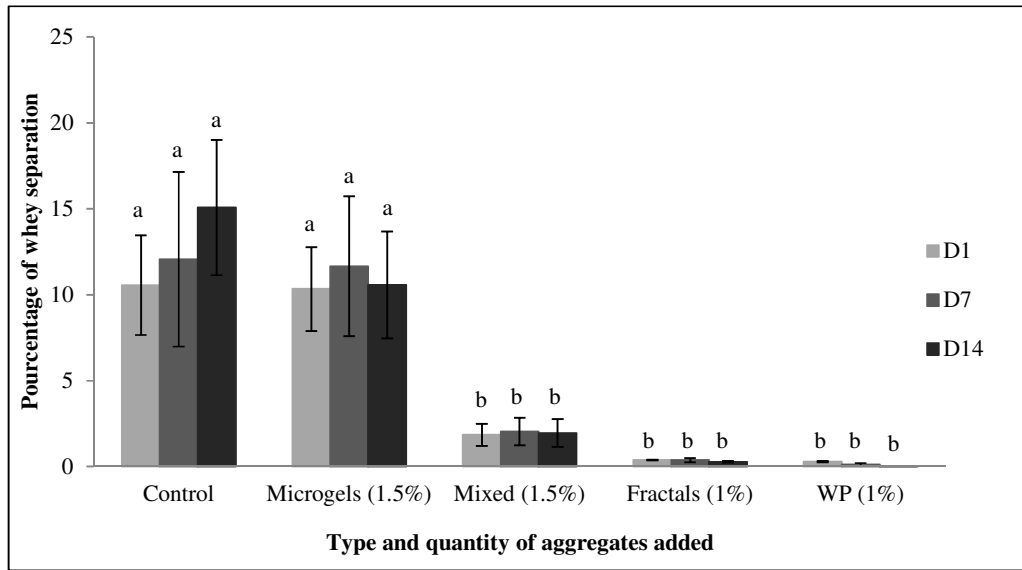


Figure 7: Change of percentage of whey separation according to type and quantity of added proteins (0 for control, 1% for fractals and WP and 1.5% for microgels and mixed aggregates) and time (days 1, 7 and 14)

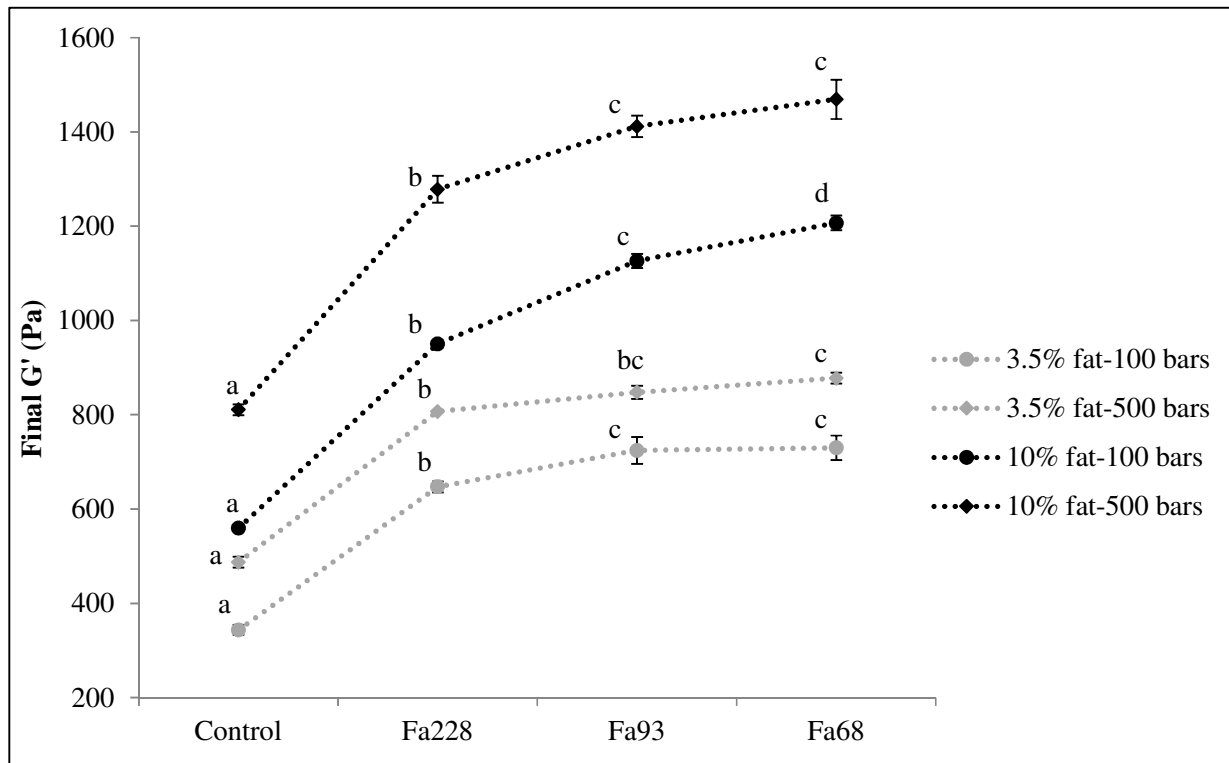


Figure 8: Storage modulus after 6 hours of acidification of control acid gels in presence of 1% fractal aggregates of different sizes (Fa228, Fa93 or Fa68) at two homogenization pressure (100 and 500 bars, 3.5% or 10% AMF)

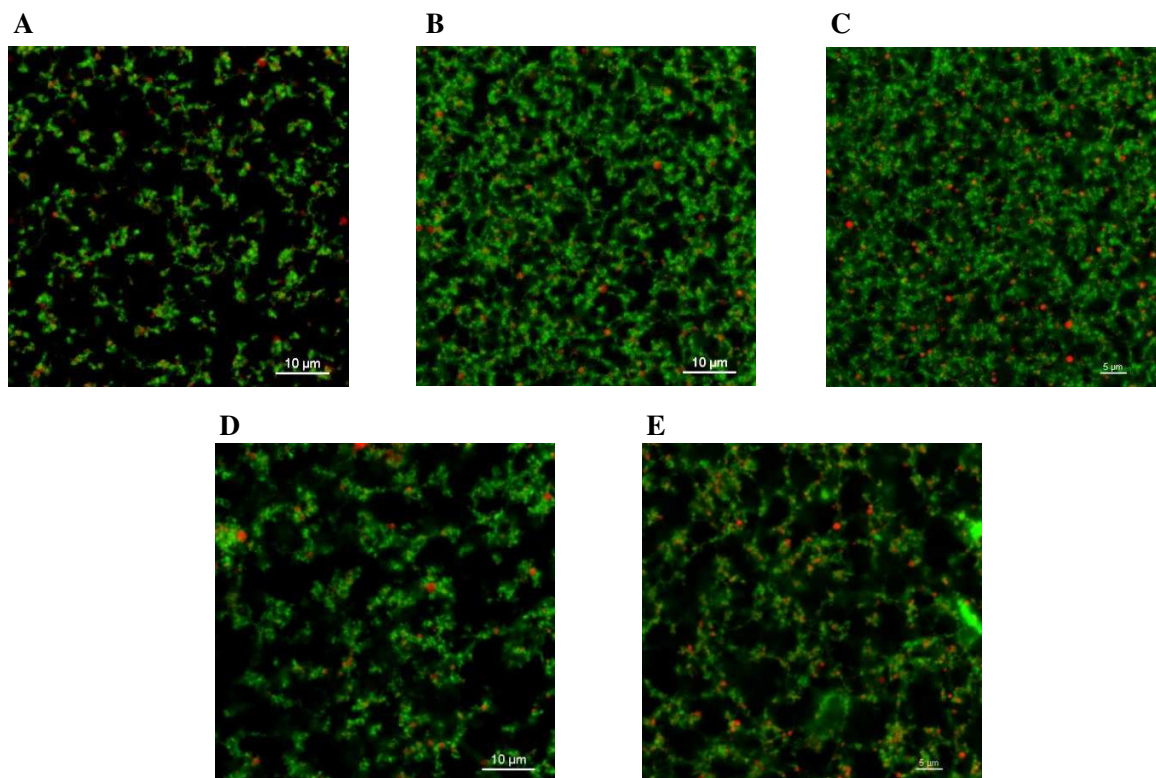


Figure 9: Confocal microscopy pictures of acid milk gels containing 3.5% AMF (A: control, B: 1% whey proteins, C: 1% fractal aggregates, D: 1% microgel aggregates and E: 1% mixed aggregates, homogenization pressure 100 bars) stained with Nile Red (for lipids) and Alexa 488 (for proteins)

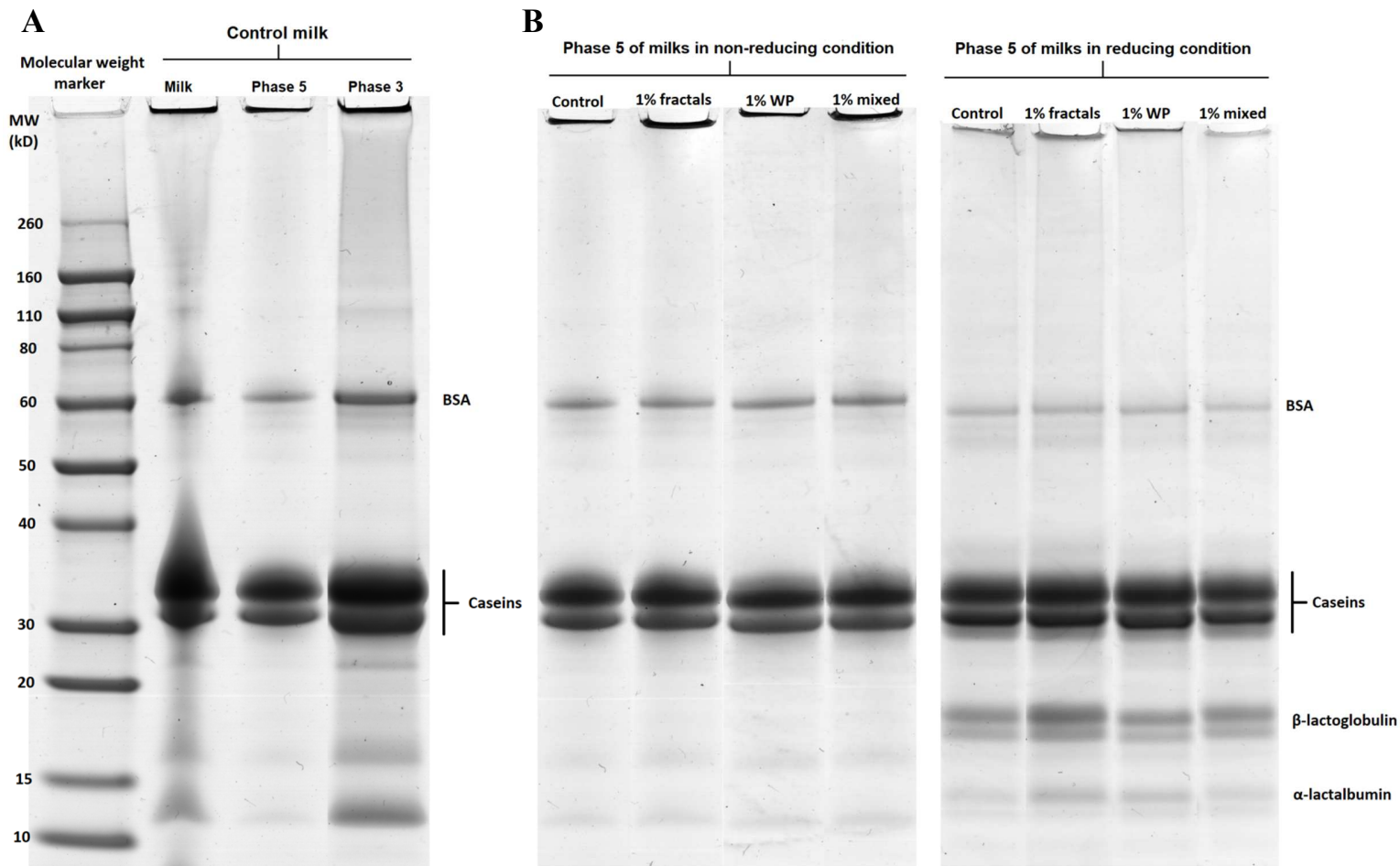


Figure 10: (A) SDS-PAGE of the interfacial composition of control milk with 10% AMF in non-reducing condition. (B) SDS-PAGE of the interfacial composition of phase 5 (containing the desorbed proteins from the interface) of control milk with 10% AMF and 1% fractals, WP or mixed aggregates in non-reducing and reducing conditions

Table 1: Low heat milk powder composition

| <b>Composition</b>                          | <b>Percentage</b> |
|---|-------------------|
| Humidity                                    | 3.4%              |
| Fat   | 0.7%              |
| Proteins/DDE* (Nx6.38)                      | 38.6%             |
| Lactose                                     | 56%               |
| Ashes                                       | 8.5%              |
| WPNI ( <i>Whey Protein Nitrogen Index</i> ) | ≥ 6 mg/g          |

\*DDE: defatted dry extract

Table 2: Average results and standard derivation for rheological and pH measurements on control acid milk gels without or with 3.5% AMF and acid milk gels with 1% added whey proteins or fractal, microgel or mixed aggregates (acidification 6h after GDL addition at 40 °C)

| <b>Sample</b>        | <b>Gelation time (min)</b> | <b>Gelation pH</b>      | <b>Tan <math>\delta</math> amplitude</b> | <b>Final G' (Pa)</b>    | <b>G' slope (FS)</b>      |
|----------------------|----------------------------|-------------------------|--|-------------------------|---------------------------|
| Sample without AMF   |                            |                         |  |                         |                           |
| <b>Control</b>       | <b>29<sup>c</sup></b>      | <b>5.44<sup>a</sup></b> | <b>0.52<sup>d</sup></b>                  | <b>234<sup>a</sup></b>  | <b>0.164<sup>d</sup></b>  |
| Standard deviation   | 1.3                        | 0.02                    | 0  | 8.6                     | 0.001                     |
| <b>1% WP</b>         | <b>22.4<sup>ab</sup></b>   | <b>5.55<sup>b</sup></b> | <b>0.37<sup>b</sup></b>                  | <b>547<sup>c</sup></b>  | <b>0.142<sup>b</sup></b>  |
| Standard deviation   | 0.2                        | 0.01                    | 0.01                                     | 24                      | 0.003                     |
| <b>1% fractals</b>   | <b>19.6<sup>a</sup></b>    | <b>5.62<sup>b</sup></b> | <b>0.33<sup>a</sup></b>                  | <b>390<sup>b</sup></b>  | <b>0.129<sup>a</sup></b>  |
| Standard deviation   | 2.3                        | 0.03                    | 0.01                                     | 81                      | 0.001                     |
| <b>1% microgels</b>  | <b>33.6<sup>d</sup></b>    | <b>5.42<sup>a</sup></b> | <b>0.51<sup>d</sup></b>                  | <b>285<sup>ab</sup></b> | <b>0.162<sup>cd</sup></b> |
| Standard deviation   | 0.1                        | 0                       | 0  | 6                       | 0.001                     |
| <b>1% mixed</b>      | <b>27.3<sup>bc</sup></b>   | <b>5.41<sup>a</sup></b> | <b>0.49<sup>c</sup></b>                  | <b>348<sup>b</sup></b>  | <b>0.158<sup>c</sup></b>  |
| Standard deviation   | 0.1                        | 0                       | 0  | 9                       | 0                         |
| Sample with 3.5% AMF |                            |                         |  |                         |                           |
| <b>Control</b>       | <b>29.4<sup>b</sup></b>    | <b>5.45<sup>a</sup></b> | <b>0.53<sup>d</sup></b>                  | <b>340<sup>a</sup></b>  | <b>0.150<sup>c</sup></b>  |
| Standard deviation   | 2.3                        | 0.03                    | 0.04                                     | 6.4                     | 0.003                     |
| <b>1% WP</b>         | <b>18.7<sup>a</sup></b>    | <b>5.62<sup>b</sup></b> | <b>0.37<sup>b</sup></b>                  | <b>721<sup>c</sup></b>  | <b>0.140<sup>b</sup></b>  |
| Standard deviation   | 0.1                        | 0.01                    | 0  | 41                      | 0.001                     |
| <b>1% fractals</b>   | <b>20.7<sup>a</sup></b>    | <b>5.60<sup>b</sup></b> | <b>0.35<sup>a</sup></b>                  | <b>647<sup>b</sup></b>  | <b>0.133<sup>a</sup></b>  |
| Standard deviation   | 0.9                        | 0.01                    | 0.01                                     | 12                      | 0.001                     |
| <b>1% microgels</b>  | <b>33.6<sup>c</sup></b>    | <b>5.42<sup>a</sup></b> | <b>0.50<sup>c</sup></b>                  | <b>366<sup>a</sup></b>  | <b>0.152<sup>c</sup></b>  |
| Standard deviation   | 0                          | 0.01                    | 0  | 11                      | 0                         |
| <b>1% mixed</b>      | <b>31.4<sup>bc</sup></b>   | <b>5.41<sup>a</sup></b> | <b>0.49<sup>c</sup></b>                  | <b>358<sup>a</sup></b>  | <b>0.163<sup>d</sup></b>  |
| Standard deviation   | 0.8                        | 0.02                    | 0.01                                     | 10                      | 0.002                     |

# Change of the texture of acid milk gel (3.5% AMF) according to protein aggregate type and concentration linked to their ultra-structure determined by confocal microscopy

